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Hybridization Capture of Illumina Libraries using xGEN Lockdown Probes

I. Purpose

To provide specific guidelines for Hybridization Capture of Illumina DNA libraries using xGEN Lockdown Probes.

II. Scope

All procedures are applicable to the BCGSC Library Core and the Library TechD groups.

III. Policy

This procedure will be controlled under the policies of the Genome Sciences Centre, as outlined in the Genome Sciences Centre High Throughput Production Quality Manual (QM.0001). Do not copy or alter this document. To obtain a copy see a QS associate.

IV. Responsibility

It is the responsibility of all personnel performing this procedure to follow the current protocol. It is the responsibility of the TechD Group Leader to ensure personnel are trained in all aspects of this protocol. It is the responsibility of Quality Systems to audit this procedure for compliance and maintain control of this procedure.

V. References

Document Title	Document Number
Hybridization capture of DNA libraries using xGen® Lockdown® Probes, version 3	IDT product protocol
IDT xGEN Lockdown Capture Protocol	Morin (SFU) lab protocol, version 3.1

VI. Related Documents

Document Title	Document Number
Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA samples	LIBPR.0017
Quantifying DNA Samples Using the Qubit™ 4 Fluorometer	LIBPR.0153
Manual Bead Clean Up using Ampure XP or ALINE Beads	LIBPR.0073
Nimbus-assisted 96-well PCR-enriched Library Construction for	LIBPR.0137

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Document Title	Document Number
Illumina Sequencing	
FFPE Genome 96-well Library Construction for Illumina Sequencing	LIBPR.0140
96-well Library Construction on the NIMBUS for xGEN Capture and Illumina Seq	TechD.0137

VII. Safety

All Laboratory Safety procedures will be complied with during this procedure. The required personal protective equipment includes a laboratory coat and gloves. See the material data sheet (SDS) for additional information.

VIII. Materials and Equipment

Name	Supplier	Number	Model or Catalogue #	
Fisherbrand Textured Nitrile gloves – various sizes	Fisher	270-058-53		✓
Bench Coat (Bench Protection Paper)	Fisher	12-007-186		✓
Small Autoclave waste bags 10”X15”	Fisher	01-826-4		✓
Gilson P2 pipetman	Mandel	GF-44801		✓
Gilson P10 pipetman	Mandel	GF-44802		✓
Gilson P20 pipetman	Mandel	GF23600		✓
Gilson P200 pipetman	Mandel	GF-23601		✓
Gilson P1000 pipetman	Mandel	GF-23602		✓
Neptune barrier tips 10 µL	Intersciences	LPBT10		✓
Neptune barrier tips 20 µL	Intersciences	LPBT20		✓
Neptune barrier tips 200 µL	Intersciences	LPBT200		✓
Neptune barrier tips 1000 µL	Intersciences	LPBT1000		✓
Galaxy mini-centrifuge	VWR	37000-700	✓	
VX-100 Vortex Mixer	Rose Scientific	S-0100	✓	
PCR Clean-DX	Aline Biosciences	C-1003-450		✓
Savant Speedvac Plus SC210A	Savant	SC201A	✓	
22R Microfuge Centrifuge	Beckman	22R Centrifuge	✓	
Peltier Thermal Cycler Tetrad	MJ Research	PTC-225	✓	
MAXYMum Recovery™ PCR Tubes, 0.2mL	VWR	22234-056		✓
MAXYMum Recovery™ PCR Tubes, 1.7 mL	VWR	22234-046		✓
96 rxn xGen® Lockdown® Reagents	IDT	1072281		✓
xGEN Lockdown probes	IDT	Custom		
xGen® Universal Blockers - TS Mix, 96 rxn	IDT	1075475		✓
xGen® Universal Blocking Oligo-TS-p5, 25 rxn	IDT	1016184		✓

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Name	Supplier	Number	Model or Catalogue #	
xGen® Universal Blocking Oligo BCGSC-p7_Universal Blocker	IDT	Custom		
Dynabeads M-270 Streptavidin	Invitrogen	65305		✓
NEBNext Ultra II Q5 Master Mix	NEB	M0544L		✓
Magnesium Sulfate ($MgSO_4$) Solution	NEB	B1003S		✓
Peltier Thermal Cycler	BioRad	Tetrad 2	✓	
5' IDX CAP, 10 µM	IDT	Custom		
3' IDX CAP, 10 µM	IDT	Custom		
Qiagen Buffer EB – 250ml	Qiagen	19086		✓
16 rxn xGen® Exome Research Panel v1.0	IDT	1056114		✓
96 rxn xGen® Exome Research Panel v1.0	IDT	1056115		✓
Human Cot-1 DNA, 500µg	Invitrogen	15279-011		✓
Mineral oil	Sigma	M5904		
Magnetic Stand 6 Stand	Ambion Inc.	AM10055		
Magnetic Stand - Invitrogen	Invitrogen	DYNAL		

xGEN Blocking Oligos for TruSeq libraries:

xGen® Universal Blockers - TS Mix

Blocking Oligos for PE libraries:

xGen® Universal Blocking Oligo BCGSC-p7_Universal Blocker
xGen® Universal Blocking Oligo-TS-p5

Post-Capture PCR primers:

5'IDX CAP: AATGATACGGCGACCACCG
3'IDX CAP: CAAGCAGAAGACGGCATACGAG

IX. Procedure

1. Pool libraries for xGEN Capture

1.1. Normalized Illumina library pools are used as input for the xGEN hybridization capture reaction. Capture input libraries may be constructed from the same or different starting material type (genomic DNA, cDNA, or circulating cell-free DNA), using one or more of the following protocols: TechD.0137 *96-well Library Construction on the NIMBUS for xGEN Capture and Illumina Seq*, LIBPR.0137 *Nimbus-assisted 96-well PCR-enriched Library Construction for Illumina Sequencing*, or LIBPR.0140 *FFPE Genome 96-well Library Construction for Illumina Sequencing*. Pool libraries according to adapter type (Paired End or TruSeq) into separate capture reactions.

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1.2. Prepare the capture library pool according to the supervisor's instructions. Libraries are normalized according to ng amount of library DNA (calculated without adapter content). If libraries have a remaining adapter content of >5%, determine the corrected library Qubit concentration by multiplying the total Qubit concentration times the percentage of the Agilent trace corresponding to the library region. A minimum total input amount of 500ng and a maximum of 6000ng are required per capture.

1.3. If the adapter content of the pre-capture library pool is >5%, and/or the volume of the library pool is >100µL, continue to step 2, Concentrate Library Pool and Remove Remaining Adapters. Otherwise, proceed to step 3, Combine and Dry Blocking Oligos, Cot-1, and DNA Library Pool.

1.4. Hybridization and Capture liquid waste should be collected in a container labeled 'IDT xGen Lockdown Capture Waste', see example label below:

Product Name: IDT xGen Lockdown Capture Waste
WHMIS Hazard Class: ☐ A: Compressed Gas
☐ B: Flammable/Combustible ☐ C: Oxidizing
☒ D: Poisonous & Infectious ☒ Immediate ☒ Other
☐ Biohazard Infectious ☒ E: Corrosive
☐ F: Dangerously Reactive
Protective Equipment Needed: ☒ Gloves ☒ Lab coat
☒ Glasses ☐ Goggles ☐ Face shield
See MSDS: IDT xGen 10x Wash 1 Buffer
IDT xGen 2X Hybridization Buffer
IDT xGen Hybridization Buffer Enhancer

Figure 1: WHMIS Label for Lockdown Waste

2. Concentrate Library Pool and Remove Remaining Adapters (0.8:1 bead clean)

2.1. The manual bead clean removes residual adapter-dimer products from the constructed libraries and concentrates the library pool(s).

2.2. Manually measure the volume of each library pool to verify the total volume of libraies pooled on the Janus. Calculate the volume of beads required for a 0.8:1 ratio bead cleanup.

2.3. Perform a manual bead clean according to LIBPR.0073 using the following conditions:

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Bead Binding Time (min)	1st Magnet Clearing Time (min)	2x 70% EtOH Wash Vol (µL)	EtOH Air Dry Time (min)	Elution Vol (µL)	Elution Time (min)	2nd Magnet Clearing Time (min)
5	5	150	5	20	3	2

2.4. Elute the DNA in 20µL EB Buffer and carefully transfer to a new low-bind 1.7mL (Axygen MAXYmum Recovery) tube.

3. Combine and Dry Blocking Oligos, Cot-1, and DNA Library Pool

3.1. Thaw xGen Lockdown Reagent Hybridization buffers at room temperature.

Note: Inspect the tube of 2X Hybridization Buffer for crystallization of salts. If crystals are present, heat the tube at 65°C, shaking intermittently, until the buffer is completely solubilized (this may require heating for several hours).

3.2. Prepare the mixtures for lyophilization according to library type in a low-bind 1.7mL (Axygen MAXYmum Recovery) tube. The hybridization blockers are specific for each type of adapter used in the library construction process.

For **TruSeq** library pools, mix the following reagents for one capture hybridization reaction:

Reagent	Volume (µL)
Pooled TruSeq libraries (TechD.0137)	xx
Cot-1 DNA, 1µg/µL (5µg total)	5
xGen® Universal Blockers - TS Mix	2

For **PE** library pools, mix the following reagents for one capture hybridization reaction:

Reagent	Volume (µL)
Pooled PE libraries (LIBPR.0137 or LIBPR.0140)	xx
Cot-1 DNA, 1µg/µL (5µg total)	5
xGen® Universal Blocking Oligo-TS-p5	2
xGen® Universal Blocking Oligo BCGSC-p7_Universal Blocker	2

3.3. Gently finger flick/vortex the tubes and quick spin.

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3.4. Dry the contents of the tube in the Savant SpeedVac with the heat set to medium. Check every 10 minutes.

- ❖ Optional Stopping Point: After drying, tubes can be stored overnight at room temperature (15-25ºC).

4. Hybridize DNA Capture Probes with the Library

4.1. Add the following to the tube from step 3.4 and incubate at room temperature for 10 minutes:

Reagent	Volume (µL)
xGEN 2x Hybridization Buffer	8.5
xGEN Hybridization Buffer Enhancer	2.7
Nuclease-Free Water	1.8

4.2. Transfer resuspended material to a 0.2 mL low-bind PCR tube (Axygen MAXYmum Recovery) and run the "**XGENCAP**" program in the BioRad Tetrad 2 Thermal Cycler to incubate the DNA for 10 minutes at 95ºC.

XGENCAP:

- 95ºC, 10 minutes
- 65ºC, ∞

Heated lid set to **75^{0}C**

4.3. Following the DNA denaturation step at 95ºC, immediately add 4µL of the Lockdown pool of probes to the tube (3-4pmol of probes). Add the probes directly to the tube in the thermal cycler (final volume = 17µL). Vortex and briefly spin down. Bring the tube back into the thermal cycler and close lid.

4.4. Incubate the hybridization reaction at 65ºC for a minimum of 4 hours. Overnight hybridization may also be performed (add 20µL mineral oil to prevent evaporation if hybridizing overnight).

5. Prepare Wash Buffers

5.1. For a single capture reaction, dilute 10X Wash Buffers (I, II, III, and Stringent) and 2X Bead Wash Buffer to create 1X working solutions as follows:

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Concentrated Buffer	Volume Concentrated Buffer (µL)	Volume Nuclease-Free Water (µL)	Final Volume of 1x Buffer (µL)
10X Wash Buffer I	30	270	300
10X Wash Buffer II	20	180	200
10X Wash Buffer III	20	180	200
10X Stringent Wash Buffer	40	360	400
2X Bead Wash Buffer	250	250	500

5.2. Store 1X buffers at room temperature for up to 4 weeks.

5.3. For each capture reaction, preheat the following wash buffers to 65ºC in a thermoblock set to 65ºC.

400µL Stringent Wash Buffer
100µL Wash Buffer I

Preheat the buffers for at least 15 minutes before starting the wash steps in section 8.

5.4. Keep the remaining 1X buffers at room temperature.

6. Prepare Streptavidin Beads

6.1. Equilibrate Dynabeads M-270 Streptavidin beads at room temperature for approximately 30 minutes before use.

6.2. Mix the beads thoroughly by vortexing for 15 seconds.

6.3. Aliquot 75µL of beads per capture into a single 1.5mL low-bind PCR tube.

6.4. Quick spin and then place the tube in a magnetic separation rack to capture the beads. Carefully remove and discard the clear supernatant into the proper disposal container, ensuring that all of the beads remain in the tube.

6.5. Add 200µL 1X Bead Wash Buffer per 75µL aliquot of beads. Vortex for 10 seconds.

6.6. Quick spin and then place the tube in the magnetic separation rack to capture the beads. Carefully remove and discard the clear supernatant into the proper disposal container, ensuring that all of the beads remain in the tube.

6.7. Repeat wash with 1X Bead Wash Buffer.

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6.8. After removing the buffer following the second wash, add 1X the original volume of beads (75µL) of 1X Bead Wash Buffer and resuspend by vortexing.

6.9. Transfer 75µL of the resuspended beads into a new 0.2mL low-bind PCR tube for each capture reaction.

6.10. Quick spin and then place the tube in a magnetic separation rack to capture the beads. Carefully remove and discard the clear supernatant into the proper disposal container, ensuring that all of the beads remain in the tube.

Important: Proceed immediately to step 7, Bind Hybridized Target to the Streptavidin Beads. Do not allow the Dynabeads to dry out. Small amounts of residual Bead Wash Buffer will not interfere with downstream binding of the DNA to the beads.

7. Bind Hybridized Target to the Streptavidin Beads

7.1. Transfer the hybridization mixture from step 4 to the tube containing prepared Streptavidin beads (ring of beads without liquid).

7.2. Mix thoroughly by pipetting up and down 10 times.

7.3. Place the tube into the thermal cycler with the XGENCAP program temperature at 65°C for 45 minutes (with heated lid set at 75°C) to bind the DNA to the beads.

7.4. Vortex the tube for 3 seconds every 15 minutes to ensure that the beads remain in suspension, and if necessary, quick spin the tube to collect contents at the bottom of the tube.

8. Wash Streptavidin Beads to Remove Unbound DNA

8.1. **Perform 65ºC washes.**

8.1.1. Add 100µL preheated Wash Buffer I to the tube.
Important: vortex briefly to mix, and spin to collect contents at the bottom of the tube.

8.1.2. Transfer the mixture to a fresh low-bind 1.5mL tube.

8.1.3. Important: Vortex briefly.

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8.1.4. Quick spin the tube and then place the tube in the magnetic separation rack to capture the beads. Carefully remove and discard the clear supernatant into the proper disposal container, ensuring that all of the beads remain in the tube.

8.1.5. Add 200µL preheated 1X Stringent Wash Buffer and pipette up and down 10 times to mix. Important: do not create bubbles during pipetting.

8.1.6. Incubate at 65°C for 5 minutes in pre-heated thermoblock.

8.1.7. Quick spin the tube and then place the tube in a magnetic separation rack to capture the beads. Carefully remove and discard the clear supernatant into the proper disposal container, ensuring that all of the beads remain in the tube.

8.1.8. Repeat wash with 1X Stringent Wash Buffer (steps 8.1.5 – 8.1.7).

8.2. **Perform Room Temperature Washes**

Note: If more than two capture reactions are being processed at the same time, the group of 1.7mL tubes may be placed into a 50mL Falcon tube together to be vortexed at the same time following the addition of Wash Buffer I and Wash Buffer II.

8.2.1. Add 200µL room temperature 1X Wash Buffer I and vortex for 2 minutes to mix.

8.2.2. Quick spin the tube and then place the tube in a magnetic separation rack to capture the beads. Carefully remove and discard the clear supernatant into the proper disposal container, ensuring that all of the beads remain in the tube.

8.2.3. Add 200µL room temperature 1X Wash Buffer II and vortex for 1 minute to mix.

8.2.4. Quick spin the tube and then place the tube in the magnetic separation rack to capture the beads. Carefully remove and discard the clear supernatant into the proper disposal container, ensuring that all of the beads remain in the tube.

8.2.5. Add 200µL room temperature 1X Wash Buffer III. Pulse mix the tubes **gently** for 30 seconds, paying attention to the beads to ensure they do not splash up the sides of the tube beyond the halfway point.

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8.2.6. Quick spin the tube and then place the tube in the magnetic separation rack to capture the beads. Carefully remove and discard the clear supernatant into the proper disposal container, ensuring that all of the beads remain in the tube.

8.2.7. Remove the tube from the magnetic separation rack and add 26.1µL Nuclease-Free Water to resuspend the beads. Mix thoroughly by pipetting up and down 10 times.

8.2.8. Note: beads often stick to the sides of the low bind tube after 1X Wash Buffer III addition. Pipette nuclease free water along the inside of the tube to aid in the resuspension of the beads. If required, add PCR brew mix to the tube to aid in resuspension.

8.2.9. **IMPORTANT**: Post capture PCR is performed on beads.

9. Post-Capture PCR

9.1. Generate the Post Capture PCR Brew Mix calculator using LIMS:

> LIMS: Mix Standard Solution > IDT_Q5_PostCap_PCR_Brew > follow the prompts > Save Standard Solution

Retrieve both the brew barcode and reagent check list label. Place both in your lab notebook.

Post Capture PCR Brew reaction volume as follows:

Reagent	Volume (µL)
Beads with captured DNA (from step 8.2.7)	26.1
100 mM $MgSO_4$ solution	1.4
2X Q5 PCR Master mix	35
5' IDX CAP, 10µM	3.75
3' IDX CAP, 10µM	3.75
Total Volume	**70**

PCR Brew mix (43.9µL)

9.2. Briefly vortex and spin the PCR reaction mix, but ensure that the beads remain in solution.

9.3. Place the PCR tube in the MJR Tetrad Thermal Cycler and run the program "**XGENPCR**" with the heated lid set at **105ºC** to amplify exome capture products

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from reactions with IDT's xGen® Exome Research Panel v1.0 (51Mb) in 10 amplification cycles. Additional PCR amplification cycles (11-13) may be required for capture products from reactions with smaller panels; consult with your supervisor about the appropriate number of post-capture amplification cycles to use.

XGENPCR(xx)

1. 98°C 45 sec
2. 98°C 15 sec
3. 65°C 30 sec
4. 72°C 30 sec
5. (go to 2 for xx cycles)
6. 72°C 1 min
7. 4°C ∞

❖ Optional stopping point: PCR-enriched capture products may be stored at 4ºC overnight.

10. Purify Post-Capture PCR Fragments

10.1. Transfer the PCR products to an appropriately labeled 1.7mL Maxymum Recovery tube.

10.2. Add 70µL Aline PCRClean DX beads into each completed PCR reaction mix containing Streptavidin beads.

10.3. Perform a manual bead clean according to LIBPR.0073, using the following conditions and wash steps with freshly made 80% EtOH as a replacement for 70% EtOH:

Bead Binding Time (min)	1st Magnet Clearing Time (min)	2x 80% EtOH Wash Vol (µL)	EtOH Air Dry Time (min)	Elution Vol (µL)	Elution Time (min)	2nd Magnet Clearing Time (min)
5	5	150	5	25	3	2

Important: Ensure the beads are washed thoroughly after the addition of each 80% EtOH wash aliquot by vortexing or pipetting EtOH/beads mixture up and down 10 times. Remove all EtOH prior to the bead drying step.

10.4. Elute in 25µL EB buffer. Transfer eluted product to a fresh 1.7mL tube, ensuring no beads are carried over.

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11. QC Validation

11.1. Measure the concentration of the capture libraries using the Qubit™ 4 Fluorometer, according to LIBPR.0153 *Quantifying DNA Samples Using the Qubit™ 4 Fluorometer.*

11.2. Measure the average bp size of the capture libraries on the Agilent Bioanalyzer using a high sensitivity DNA (HS DNA) chip according to LIBPR.0017 *Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA samples*.

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Appendix A – LIMS tracking

1. Samples rearray – IDX pipeline, 1.5mL tube
2. IDT_Capture
3. MXC_Post_Hyb_Cleanup
4. MXC_Post_Cap_PCR. Throw out the post capture PCR template tray in LIMS.
5. Final_Submission –IPET pipeline (supervisor will advise if there is a different pipeline)

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